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Cell-free conversion of 1-deoxy-D-xylulose 5-phosphate and 2-*C*-methyl-D-erythritol 4-phosphate into β-carotene in higher plants and its inhibition by fosmidomycin

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Abstract.
The presence of the deoxyxylulose pathway in chromo- and chloroplasts of various higher plants was demonstrated by the conversion of labelled 1-deoxy-D-xylulose 5-phosphate into β-carotene and geranylgeraniol, in the presence of ATP. The antibiotic fosmidomycin inhibited the formation of terpenoids from 1-deoxy-D-xylulose 5-phosphate, whereas it did not affect the conversion of 2-*C*-methyl-D-erythritol 4-phosphate or isopentenyl diphosphate into terpenoids.

Keywords: 1-deoxy-D-xylulose 5-phosphate, 2-*C*-methyl-D-erythritol 4-phosphate, isopentenyl diphosphate, terpenoid biosynthesis

The deoxyxylulose pathway leading to terpenoids via isopentenyl diphosphate (IPP) and dimethylallyl diphosphate has been shown to be operative in certain bacteria and in all higher plants.[1-3] Feeding experiments with unphosphorylated precursors demonstrated the incorporation of 1-deoxy-D-xylulose[2] (DX) and 2-*C*-methyl-D-erythritol[4,5] (ME) into terpenoids. In addition, the first two enzymes in this novel pathway have been discovered and cloned: 1-deoxyxylulose 5-phosphate synthase[6-8] and 1-deoxy-D-xylulose 5-phosphate reductoisomerase[5] (Fig. 1). However, no direct evidence for the *in vitro* conversion of the putative phosphorylated intermediates 1-deoxy-D-xylulose 5-phosphate (DXP) and 2-*C*-methyl-D-erythritol 4-phosphate (MEP) into terpenoids has been reported.

Chromoplasts of *Capsicum annuum* L.,[9] *Narcissus pseudonarcissus* L.[10] and chloroplasts[11] of various higher plants have been found to efficiently incorporate proffered IPP into carotenoids, especially into β-carotene and geranylgeraniol. The obvious biochemical activity of these organelles encouraged us to extend these studies to the suspected intermediates of the alternative pathway, i.e. DXP and MEP.

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The availability of [1,2-^{14}C] DXP (spec. activity 2.31x10^9 Bq/mmol) synthesized from [1,2,3-^{14}C] pyruvate and glyceraldehyde 3-phosphate by the cloned synthase[6] and chemically synthesized[12] [1-^{3}H] MEP (spec. activity 10.7x10^{10} Bq/mmol) made it possible to study the potential incorporation of both suspected intermediates into β-carotene. [1-^{14}C] IPP (spec. activity 2.08x10^9 Bq/mmol) from Amersham Pharmacia served as a control. *Capsicum* chromoplasts (2 mg of protein) were incubated in the standard incubation mixture[9] containing 100 mM Hepes buffer pH 7.6, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 2mM NADP, 20 μM FAD, 5 mM NaF, 6 mM ATP, 2 mM NADPH and 1.8 nmol IPP (3.7x10^3 Bq) in a final volume of 500 μl for 15 hrs at 30°C. The reaction was terminated by extracting the incubation mixture twice with 1 ml of chloroform. The combined organic phases were taken to incipient dryness, and an aliquot was subjected to scintillation counting. Quench corrections were made by the internal standard method. The remaining chloroform extract was subjected to TLC and either developed with hexane-diethyl ether = 6:4 (v/v): the carotene fraction (R_f 0.96) is well separated from geranylgeraniol (R_f 0.35) or with light petroleum-toluene = 9:1 (v/v) (β-carotene R_f 0.47). The identity of β-carotene was confirmed by MS-EI at 70 eV (*m/z*: 536). Under these conditions, labelled IPP yielded 28% incorporation into carotenoids and 2% into geranylgeraniol in agreement with published data.[9]

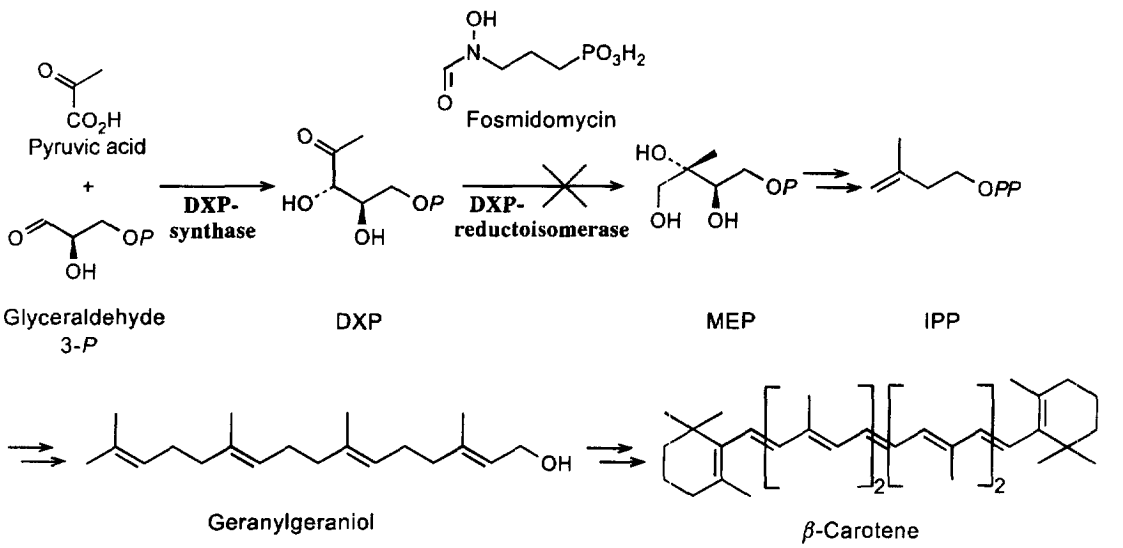

Fig. 1 Deoxyxylulose pathway for terpenoid biosynthesis showing inhibition of DXP-reductoisomerase by fosmidomycin.

Replacement of IPP by [1,2-^{14}C] DXP (3.7x10^3 Bq) gave 19% incorporation into carotenoids and 5% incorporation into geranylgeraniol while [1-^{3}H] MEP (5.55x10^4 Bq) yielded 12% incorporation into carotenoids and 2% incorporation into geranylgeraniol. Maximal conversion

occurred in the presence of 5-6 mM ATP. Omission of ATP completely blocked the conversion of both labelled DXP and MEP into the terpenoids. Although NADP, FAD and NADPH were added to the incubation mixture,[9] their direct involvement in the formation of carotenoids could not be shown. NADPH is known to be a cofactor for the conversion of DXP to MEP by the reductoisomerase[5] and may be available in the chromoplast suspension at saturating concentration. Similar experiments conducted with *Narcissus* chromoplasts[10] again demonstrated the conversion of [1,2-^{14}C] DXP into carotenoids (5%) and geranylgeraniol (2%). Incubation of chloroplasts from *Capsicum annuum* fruits with [1-^{14}C] IPP yielded an incorporation of 20% into the carotenoid fraction, [1,2-^{14}C] DXP gave an incorporation of 21%, whereas [1-^{3}H] MEP gave only about 2%. Similar results were obtained with chloroplasts from *Lactuca sativa* L. ssp. *capitata* (L.) ALEF., *Avena sativa* L., *Spinacia oleracea* L., *Brassica oleracea* L. ssp. *oleracea* convar. *botrytis* (L.) ALEF. var. *italica* and others. It has been shown here for the first time that DXP and MEP are true precursors of higher plant terpenoids such as geranylgeraniol and β-carotene. Furthermore, it was demonstrated that chloro- and chromoplasts have the complete enzymatic outfit to convert distant precursors like DXP and MEP to higher terpenoids such as tetraterpenes.

Recently it has been shown that the antibiotic fosmidomycin (3-(*N*-formyl-*N*-hydroxyamino)propylphosphonic acid) acts as a specific inhibitor of the DXP-reductoisomerase[13] of *Escherichia coli* (Fig. 1). Since this inhibitor is known also to be a potent herbicide,[14] we followed the conversion of labelled IPP, DXP and MEP in the presence and absence of varying concentrations of fosmidomycin into carotenoids using the *Capsicum* chromoplast system. Fosmidomycin at 100 µM concentration completely inhibited the conversion of [1,2-^{14}C] DXP into β-carotene. Fosmidomycin inhibited the formation of β-carotene from DXP in a dose-dependent manner with an IC_{50} value of 2.5 µM. If, however, labelled IPP and MEP were used as substrate in the presence of even 100 µM of the antibiotic in the chromoplast system, absolutely no inhibition of conversion into β-carotene was observed. This excluded the previous suggestion that fosmidomycin may inhibit prenyltransferases in the β-carotene pathway.[15] To verify this highly specific inhibition a double label experiment was conducted. [1,2-^{14}C] DXP ($3.7x10^3$ Bq) and [1-^{3}H] MEP ($5.55x10^4$ Bq) were incubated in the standard assay system (above). Carotenoids were isolated and showed a double labelling pattern of 33 330 dpm ^{14}C and 256 500 dpm ^{3}H (1:7.7). The same experiment conducted in the presence of 100 µM fosmidomycin yielded carotenoids containing 80 dpm ^{14}C and 228 900 dpm ^{3}H (1:2861). This indicates that DXP was completely prevented from conversion to β-carotene by the inhibitor while MEP was not. Final proof that only the DXP-reductoisomerase was inhibited came from a study of the phosphorylated intermediates using an established[16] ion-pair HPLC system. [1,2-^{14}C] DXP in the standard incubation mixture (above) yielded 8.5% of unconverted DXP (R_t = 18.6 min), 12.8% MEP (R_t = 13.2 min) and the dephosphorylated intermediates DX and ME (R_t = 5.2 min) 12.7%. The same incubation, however, in the presence of 100 µM fosmidomycin yielded absolutely no MEP but a slightly higher amount of dephosphorylated DX (18.9%):

From these results it is obvious that fosmidomycin in higher plants specifically inhibits the DXP-reductoisomerase of the deoxyxylulose pathway. This enzyme should, therefore, be

considered as a novel target for herbicide action. The chromo- and chloroplast system may be useful for the screening of postulated inhibitors of the deoxyxylulose pathway.

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